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ORIGINAL CONTRIBUTION

Increased cardiovascular risk in rats with primary renal dysfunction; mediating role for vascular endothelial function

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Abstract Primary chronic kidney disease is associated with high cardiovascular risk. However, the exact mechanisms behind this cardiorenal interaction remain unclear. We investigated the interaction between heart and kidneys in novel animal model for cardiorenal interaction. Normal Wistar rats and Munich Wistar Fromter rats, spontaneously developing renal dysfunction, were subjected to experimental myocardial infarction to induce cardiac dysfunction (CD) and combined cardiorenal dysfunction (CRD), respectively ($N = 5$–10). Twelve weeks later, cardiac- and renal parameters were evaluated. Cardiac, but not renal dysfunction was exaggerated in CRD. Accelerated cardiac dysfunction in CRD was indicated by decreased cardiac output (CD 109 ± 10 vs. CRD 79 ± 8 ml/min), diastolic dysfunction (E/e') (CD 26 ± 2 vs. CRD 50 ± 5) and left ventricular overload (LVEDP CD 10.8 ± 2.8 vs. CRD 21.6 ± 1.7 mmHg). Congestion in CRD was confirmed by increased lung and atrial weights, as well as exaggerated right ventricular hypertrophy. Absence of accelerated renal dysfunction, measured by increased proteinuria, was supported by absence of additional focal glomerulosclerosis or further decline of renal blood flow in CRD. Only advanced peripheral endothelial dysfunction, as found in CRD, appeared to correlate with both renal and cardiac dysfunction parameters. Thus, proteinuric rats with myocardial infarction showed accelerated cardiac but not renal dysfunction. As parameters mimic the cardiorenal syndrome, these rats may provide a clinically relevant model to study increased cardiovascular risk due to renal dysfunction. Peripheral endothelial dysfunction was the only parameter that correlated with both renal and cardiac dysfunction, which may indicate a mediating role in cardiorenal interaction.

Keywords Cardiorenal · Congestive heart failure · Endothelial dysfunction

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Introduction

Even mild renal dysfunction substantially increases cardiovascular risk [12, 20, 28]. Moreover, many patients with heart failure suffer from concomitant renal dysfunction [19], indicating a strong cardiorenal interaction. Although well recognized, the pathophysiology of this interaction is largely unclear. Patients characterized by a condition of primary chronic kidney disease [36] are at an extremely high cardiovascular risk; 10–20-fold increased risk of cardiac death; the 2 year mortality rate after myocardial infarction is

increased up to 50%, compared to 10 year mortality in the general population of 25% [17]. These findings have revived interest in the interrelation between heart and kidneys.

To investigate the interaction between heart and kidneys in this high-risk group of patients, different animal models have been used so far [41]. Most studies include rats with either unilateral [48] or 5/6 nephrectomy [9, 55] to induce (a predisposition for) renal dysfunction, that were subjected to chronic experimental heart failure. Although studies have presented accelerated renal dysfunction [48], in neither study accelerated cardiac dysfunction has been observed. Only one study reported worsening of cardiac dysfunction and exaggerated cardiac remodeling, but this could well be attributed to the larger infarcts in the cardiorenal group [10]. As all models have their advantages and disadvantages, the acute character of the nephrectomy in the above models and the little amount of renal tissue left for therapeutic intervention, provides a clear disadvantage in the translation of the results to the human cardiorenal syndrome. We aimed to study cardiorenal interaction in an alternative animal model, better mimicking the clinically high-risk condition; rats that spontaneously develop slowly progressive renal dysfunction, subjected to experimental heart failure. Parameters of renal as well as cardiac dysfunction were recorded and correlated to study interaction between heart and kidneys. As heart and kidneys are connected by vasculature, and endothelial function is reported to predict outcome in cardiac as well as renal dysfunction, vascular endothelial dysfunction was studied as potential mechanism underlying cardiorenal interaction.

Methods

Animals

The study was performed in male Munich Wistar Fromter rats (MWF/ZtmHsd) and age-matched Wistar rats (HsdCpb:WU), as their genetic background (Harlan, The Netherlands/USA). Animals were housed in groups under standard conditions at 12 h light/dark cycle at the animal facilities of the University of Groningen. All animals were fed standard diet (standard rat chow, Hope Farms, Woerden, The Netherlands) and received food and water ad libitum. All experiments were conducted in accordance with the NIK Guide for the Care and Use of Laboratory Animals and were approved by the Committee for Animal Experiments of the University of Groningen.

Experimental protocol

At 12 weeks of age, all rats were placed in metabolic cages for measurements of water and food intake and 24 hour urine collection. Subsequently, under 2.5% isoflurane anaesthesia blood samples (±1.5 ml) were withdrawn from the tail vein. Urine and plasma samples were processed and stored at −80°C for later analyses. Subgroups of Wistar and MWF rats ($n = 5$ each) were sacrificed prematurely to obtain baseline measurements of cardiac function and to collect cardiac and renal tissue for further analysis. The remaining animals were allowed 1 week recovery before they were subjected to permanent coronary artery occlusion or sham surgery as described before [52]. Five rats of each group (ECHO apparatus became available half way through the study) underwent echocardiography at baseline and at 3, 7 and 10–11 weeks after surgery to evaluate the time course of cardiac function. At the same time points, plasma and urine were collected from all rats (metabolic cages) to examine the time course of renal (dys)function.

Twelve weeks after surgery, measurements of left ventricular function and cardiac and renal hemodynamics were obtained invasively. This time point was chosen because of anticipated endothelial dysfunction in CD animals [13]. Blood samples were collected from the tail vein and processed for further analyses. The heart was dissected, weighted and cooled in ice-cold saline for diastolic arrest. A mid-ventricular slice was processed for histological analysis. The remaining tissue was separated in right ventricle, interventricular septum, viable- and infarcted left ventricle, frozen in liquid nitrogen and stored at −80°C.

Lung tissue was weighted. Kidneys were dissected, weighted and processed for histological analysis. The thoracic aorta was dissected and cut in rings for in vitro endothelial function analysis [44], as well as structural analysis.

Echocardiography

Echocardiographical measurements were obtained with the use of a Vivid 7 (GE Healthcare) equipped with a 10 MHz transducer. Under isoflurane anaesthesia (2.5%), rats were placed on a heating pad to keep body temperature at 37°C. Images in parasternal long-axis, short-axis and four-chamber apical view were obtained. The ejection fraction (EF) was calculated using the Teichholz method and cardiac output (CO) was estimated from the aortic flow profile and the diameter at valvular levels as follows: CO = aortic valve area times aortic velocity time integral times heart rate, as described before [45]. Mitral inflow measurement of early filling velocity (E) was obtained from an apical four-chamber view using pulsed Doppler with the sample placed at the tips of mitral leaflets. Tissue Doppler imaging measurement of mitral valve septal annular velocity (e') was also obtained from the four-chamber apical view. All

calculated parameters have been presented as the mean of five consecutive beats to avoid beat-to-beat variation. As echocardiography could have been obtained only in subgroups, results should be regarded as indication, rather than as group evaluation with statistical analysis.

Blood and urine measurements

After urine collection, blood samples were obtained under isoflurane anaesthesia. Measurements of plasma creatinine, electrolytes, haemoglobin, and haematocrit levels were performed with an iSTAT handheld analyser (ABBOTT) and appropriate cartridges (Creatinine; EG7+). Standard blood count was performed in whole blood samples. For that, whole blood sampled from the tail vein was transported immediately to the hospital. Remaining blood was processed and plasma was stored at −80°C for later analyses.

PRA was determined by Gammacoat RIA kit (Diasorin, Minnesota, USA) according to the manufacturer's instructions and expressed as ngAngI/ml/h.

BNP was measured with the use of BNP-45 EIA kit (Phoenix Pharmaceuticals, Inc).

Total urinary protein levels were determined using TCA precipitation measurement (Nephelometer analyzer II, Dade Behring, Marburg, Germany).

All measurements were performed according to the manufacturer's instructions.

Hemodynamics and cardiac function

Under isoflurane anaesthesia, hemodynamics and cardiac performance were measured using a pressure transducer catheter (Micro-Tip 3French, Millar Instruments Inc., Houston, TX) inserted through the right carotid artery. The catheter was positioned in the aortic root and mean arterial blood pressure (MAP) and heart rate (HR) were measured. The catheter was advanced into the left ventricle, and left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were recorded. The maximal rates of increase and decrease in left ventricular pressure ($+dP/dt_{max}$ and $-dP/dt_{max}$) were determined as parameters of myocardial contractility and relaxation, respectively. Then, the catheter was withdrawn and immediately positioned in the vena cava superior just above the heart, and central venous pressure (CVP) was measured and total peripheral resistance (TPR) was calculated as TPR = MAP − CVP/CO.

The abdominal cavity was opened and a flow probe (Transonic, Ithaca, NY, USA) was positioned on the left renal artery [56]. After stabilization, renal blood flow (RBF) was measured. Renal vascular resistance (RVR) was calculated as RVR = MAP − CVP/RBF.

Histology

A 1.5 mm thick mid-ventricular slice was fixated in 2% phosphate buffered paraformaldehyde for at least 24 h, dehydrated, and embedded in paraffin for measurement of infarct size and morphometric analysis of cardiac structure. Deparaffinized 5 μm sections were either stained with Sirius Red and fast green to distinguish infarct from viable tissue and to visualize interstitial collagen; with Gomori's silver staining in order to visualize individual myocytes; or with Lectin GSI to visualize capillaries. Methods as well as the measurements of infarct size, myocyte size, capillary density and interstitial fibrosis are described in detail by van Kerckhoven et al. [49]. Infarct size was expressed as percentage of left ventricular circumference.

Kidneys were stained with PASS staining and scored on presence and degree of focal glomerular sclerosis as described before [44].

Aortic sections were stained with Virhoff staining. Aortic hypertrophy was obtained from medial thickness, corrected for lumen diameter, as described before [8].

Cardiomyocyte function

Force measurements were performed in single, mechanically isolated cardiomyocytes as described previously [27, 50]. Briefly, tissue samples were defrosted in relaxing solution, mechanically disrupted and incubated in relaxing solution supplemented with 0.5% Triton X-100 to remove all membrane structures. Single cardiomyocytes were attached with silicone adhesive between a force transducer and a motor. Sarcomere length of isolated cardiomyocytes was adjusted to 2.2 μm. Isometric force was measured in maximally activating calcium solution ($pCa = -_{10}\log[Ca^{2+}] = 4.5$) and in relaxation solution (pCa 9.0) to determine maximal force generating capacity (F_{max}) and passive force (F_{pas}), and normalized for cardiomyocyte cross-sectional area. On transfer of the cardiomyocyte from relaxing to activating solution, isometric force started to develop. Once a steady state force level was reached, the cell was shortened within 1 ms to 80% of its original length to determine the baseline of the force transducer. The distance between the baseline and the steady force level is the total force (F_{total}). The cell was re-stretched and returned to the relaxing solution, in which a second slack test, of 10 s duration was performed to determine passive force. Maximal force was obtained by subtracting passive force from the total force, i.e. $F_{max} = F_{total} - F_{pas}$.

Endothelial function

Maximal endothelium-dependent relaxation was measured in vitro in aortic rings obtained from subgroups of the

experimental groups, as described before [44]. Briefly, thoracic aorta segments (approximately 2 mm) were cleaned of adherent tissue and mounted in an organ bath with Krebs solution (pH 7.5) containing (in mmol/L): NaCl (120.4), KCl (5.9), $CaCl_2$ (2.5), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), glucose (11.5), $NaHCO_3$ (25.0) which was kept at 37°C and continuously bubbled with 95% O_2 and 5% CO_2. After equilibration, viability of smooth muscle cells and endothelium was tested using phenylephrine (PE; 10^{-6} mol/L). After wash out and another 30 min of stabilization, endothelial function was measured as endothelium-dependent relaxation to cumulative concentration of acetylcholine (ACh; $3 \times 10^{-8} - 3 \times 10^{-4}$ mol/L) in the vessels precontracted by PE (10^{-6} mol/L). Area under the curve (AUC) was obtained as measure for endothelial function. Finally, sodium nitroprusside (10^{-3}mol/L) was added to the organ baths to obtain endothelium-independent relaxation. In order to examine the role of cyclo-oxygenase (COX) [44], Ach-induced relaxation was studied in parallel in separate aortic rings in the presence of either 10^{-6} M indomethacin or 10^{-5} nimsulin, to inhibit COX1 and COX2, or only COX2, respectively.

Aortic sections are processed for analysis of endothelial nitric oxide synthase (e-NOS) expression using RT-PCR. For that, total RNA was isolated using TRIzol reagent (Invitrogen Corporation, Breda, The Netherlands) and converted to cDNA by QuantiTect Reverse Transcription (Qiagen, Venlo, The Netherlands). Gene expression was measured with Absolute QPCR SYBR Green ROX Mix (Abgene, Epsom, UK) in the presence of 7.5 ng cDNA and 200 nM forward and reverse primers. qRT-PCR was conducted on the Biorad CFX384 (Biorad, Veenendaal, The Netherlands). Initial denaturation and activation of the DNA polymerase at 95°C for 3 min was followed by 35 cycles with denaturation for 15 s at 95°C and annealing and elongation for 30 s at 60°C, followed by a melt curve. Gene expression levels were corrected for ribosomal protein large (Rplp0). Primers used were endothelial NO synthase (eNOS) forward TCCTAACTTGCCTTGCATCC, eNOS reverse GGCAGCCAAACACCAAAGTC, Rplp0 forward CCTCATACCAGCGACGATTC, Rplp0 reverse ATGTGGAGGAGTCTCACTTC.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data of rats with infarct <20% of the left ventricle were excluded from analysis, as these small infarcts are hemodynamically fully compensated [37]. Analyses were performed using SPSS version 16. Parameters were compared using 2-way analysis of variances (ANOVA); effects of cardiac dysfunction as well as renal dysfunction were analyzed first separately, followed by analysis of interaction. This is presented in the figures using different symbols. In case data were not normally distributed, a non-parametric Kruskal–Wallis test followed by a Mann–Whitney *U* test with correction for multiple comparisons was used. Differences were considered significant at the level of 0.05.

Echocardiography could be obtained in only 3–5 rats per group, and hence results should be regarded as indication, rather than as group evaluation with statistical analysis.

Results

Control rats

Twelve weeks old rats from both strains (MWF = RD; Wistar = control) were sacrificed to obtain baseline values, and general characteristics are presented in Table 1. Food and water intake did not differ between RD rats and controls, nor did urine production. RD rats had a lower body weight than age-matched control. Lung weight and

Table 1 Characterization of control (Wistar) and RD (MWF) rats at 12 weeks of age

Group	Control	RD
N	5	5
BW (g)	386 ± 7	249 ± 14[a]
Food intake (g/24 h)	15 ± 4	19 ± 1
Water intake (ml/24 h)	24 ± 3	26 ± 2
Urine production (g/24 h)	15 ± 2	11 ± 1
HW/BW (mg/g)	3.2 ± 0.2	4.5 ± 0.5[a]
Lung/BW (mg/g)	3.3 ± 0.0	4.8 ± 0.2[a]
RKW/BW (mg/g)	3.7 ± 0.1	3.7 ± 0.1
UPE (mg/24 h)	28 ± 5	76 ± 19[a]
FGS (AU)	0.4 ± 0.4	1.6 ± 0.4[a]
Plasma creat (mg/L)	31 ± 1	25 ± 4
MAP (mmHg)	89 ± 4	102 ± 3[a]
LVSP (mmHg)	117 ± 5	131 ± 3[a]
LVEDP (mmHg)	5.3 ± 2.4	6.2 ± 1.9
HR (beats/min)	359 ± 18	364 ± 14
CVP (mmHg)	1.2 ± 1.9	2.1 ± 1.7
RBF/KW (ml/min/g)	6.0 ± 1.6	2.6 ± 0.6[a]
Hct (%)	47.6 ± 0.4	44.2 ± 0.3[a]

HW heart weight, *BW* body weight, *RKW* right kidney weight, *UPE* urinary protein excretion, *FGS* focal glomerulosclerosis, *Creat* creatinine levels, *MAP* mean arterial pressure, *LVSP* left ventricular systolic pressure, *LVEDP* left ventricular enddiastolic pressure, *HR* heart rate, *CVP* central venous pressure, *RBF* renal blood flow, *Hct* hematocrit

[a] Significant difference between control and RD

Table 2 General characteristics of the experimental groups at 25 weeks of age

Group	Control	CD	RD	CRD
N	10	6	9	10
BW (g)	482 ± 11	466 ± 14	340 ± 9[b]	342 ± 8[b]
Food intake (g/24 h)	11 ± 2	13 ± 3	13 ± 1	11 ± 1
Water intake (ml/24 h)	21 ± 1	26 ± 5	22 ± 1	22 ± 1
Urine production (g/24 h)	15 ± 2	18 ± 3	13 ± 1	14 ± 1
Hct (%)	47.8 ± 0.6	48.7 ± 1.0	45.0 ± 0.8[b]	45.7 ± 0.7[b]
Plasma [Na] (mmol/L)	137 ± 1	138 ± 1	137 ± 0	136 ± 1
Plasma [K] (mmol/L)	4.4 ± 0.2	4.7 ± 0.2	4.5 ± 0.1	4.7 ± 0.1
Plasma [Cl] (mmol/L)	102 ± 1	103 ± 1	105 ± 1	104 ± 0
Urine [Na] (mmol/L)	92 ± 17	123 ± 36	147 ± 11[b]	159 ± 22[b]
Urine [K] (mmol/L)	115 ± 15	125 ± 23	153 ± 9[b]	152 ± 21[b]
Urine [Cl] (mmol/L)	101 ± 20	136 ± 40	176 ± 13[b]	184 ± 25
RKW/BW (mg/g)	3.7 ± 0.1	3.3 ± 0.1[a]	3.3 ± 0.1[b]	3.1 ± 0.1[b]
FGS (AU)	2.4 ± 0.6	3.6 ± 1.2	22.4 ± 2.1[b]	12.1 ± 1.4[b]

BW body weight, *Hct* hematocrit, *HW* heart weight, *RKW* right kidney weight, *FGS* focal glomerulosclerosis, *MI* myocardial infarction, *CD* cardiac disease, *RD* renal disease, *CRD* cardiorenal disease

[a] Significant effects of cardiac dysfunction

[b] Significant effect of renal dysfunction

heart weight corrected for body weight were significantly increased in RD. At 12 weeks of age, renal dysfunction was confirmed by increased urinary protein excretion (UPE) and focal glomerulosclerosis (FGS) in RD, but no increase in plasma creatinine levels was observed. RD rats had slightly higher blood pressures, reflected in a 29% increase in left ventricular myocyte cross-sectional area (NS), with normal heart rate and normal left (LVEDP) and right (CVP) cardiac filling pressures. Renal blood flow, however, was strongly reduced in RD due to elevated renal vascular resistance (47 ± 12 vs. 17 ± 4 mmHg/ml/min, in RD and control, respectively). Hematocrit was significantly lower in RD rats.

Apart from a slightly increased myocyte size, histological analysis showed no signs of adverse left ventricular remodeling in RD; myocyte cross-sectional area 535 ± 71 versus 414 ± 48 μm^2; interstitial fibrosis: 5.0 ± 0.9 versus 5.9 ± 0.5%; capillary density: 2,891 ± 337 versus 2,156 ± 141 #/mm^2; number of capillaries per cardiomyocyte 1.92 ± 0.28 versus 1.14 ± 0.19, in RD versus control, respectively.

Myocardial infarction

Cardiac dysfunction was induced by experimental myocardial infarction (MI). This procedure resulted in 22% mortality, which occurred mainly within the first 24 h after surgery. Overall infarct size was not different in both groups (33 ± 5% in CD and 34 ± 4% in CRD), neither after exclusion of 3 rats with infarcts <20% (34 ± 5% in CD and 38 ± 3% in CRD).

Characteristics of experimental groups at 25 weeks are presented in Table 2. Strain-related characteristics as seen at 12 weeks (Table 1) remained present in sham groups at 25 weeks. Whereas plasma concentrations of electrolytes did not differ between groups, concentrations in urine were significantly higher in rats with RD. MI had no effect on electrolyte concentrations in urine.

Renal effects

Time course of UPE is shown in Fig. 1. As anticipated, RD rats showed progressive proteinuria with age, which was absent in controls and CD rats. When renal dysfunction was combined with cardiac dysfunction in CRD, besides an initial decrease, proteinuria progressed at the same rate as in RD. The initial decrease occurred in both MI groups, CD and CRD, at the same percentage. In RD, FGS increased with age from 1.6 ± 0.4 AU at 12 weeks to 22.4 ± 2.1 AU

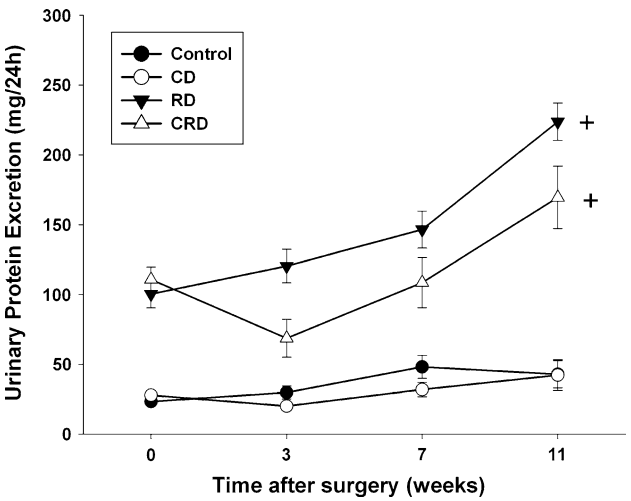

Fig. 1 Time course of urinary protein excretion in the experimental groups. *Control* ($n = 10$), *CD* cardiac disease ($n = 6$), *RD* renal disease ($n = 6$), *CRD* cardiorenal disease ($n = 10$). [+]Significant effect of renal dysfunction

Table 3 Systemic and cardiac hemodynamics measured at 25 weeks of age

Group	Control	CD	RD	CRD
N	10	6	9	10
HR (b/min)	331 ± 14	336 ± 35	360 ± 28	416 ± 38
MAP (mmHg)	94 ± 3	88 ± 4	107 ± 4[b]	84 ± 5[a]
LVSP (mmHg)	128 ± 4	123 ± 8	134 ± 3	108 ± 2[ac]
+dP/dt (mmHg/s)	9,091 ± 299	7,463 ± 375[a]	8,993 ± 318	8,452 ± 455[a]
−dP/dt (mmHg/s)	7,463 ± 375	6,009 ± 432[a]	6,199 ± 513	4,377 ± 299[ac]
RBF/KW (ml/min.g)	7.1 ± 0.8	6.7 ± 0.4	2.8 ± 0.7[b]	2.7 ± 0.5[b]

CD cardiac disease, *RD* renal disease, *CRD* cardiorenal disease, *HR* heart rate, *MAP* mean arterial pressure, *LVSP* left ventricular systolic pressure, ±*dP/dt* positive/negative first derivative of pressure signal, *RBF/KW* renal blood flow/kidney weight

[a] Significant effects of cardiac dysfunction

[b] Significant effect of renal dysfunction

[c] Significant effect of combined cardiorenal dysfunction

at 25 weeks, and closely matched with the progression of proteinuria, with no additional increase due to concomitant cardiac dysfunction. In fact, there was a highly significant, positive correlation between UPE and FGS ($r^2 = 0.64$, $p < 0.001$). RBF was significantly lower in RD and was not affected by MI in either strain (Table 3). RVR was significantly higher in RD rats than in controls (52.8 ± 13.1 and 13.2 ± 1.9 mmHg/ml/min, respectively), but not significantly affected by MI (CRD 39.5 ± 6.9 mmHg/ml/min; CD 13.5 ± 1.3 mmHg/ml/min). This hemodynamic renal parameter RVR was significantly, negatively correlated with both structural (FGS; $r^2 = 0.473$, $p < 0.001$) and functional (UPE; $r^2 = 0.328$, $p = 0.002$) renal parameters.

Creatinine clearance was similar in all groups (control 8.2 ± 0.9; CD 7.3 ± 0.2; RD 10.0 ± 1.3 and CRD 9.1 ± 1.1 ml/min/kg) and decreased approximately 25% with aging irrespective of absence/presence of MI. No significant correlations of creatinine clearance with other renal parameters were found.

Cardiac effects

Effects on cardiac function

Time course of cardiac dysfunction is presented in Fig. 2a and b. The EF (Fig. 2a) was 85% in sham rats and remained stable over time. At 3 weeks, EF was severely

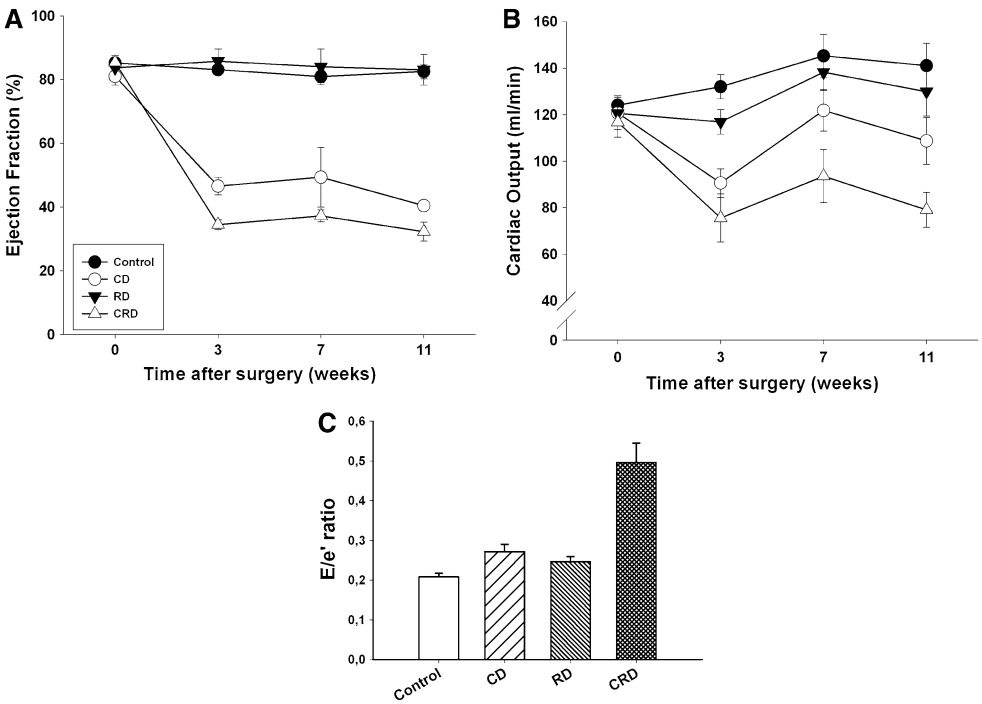

Fig. 2 Cardiac function parameters obtained by echocardiography. *E/e′* early filling velocity (*E*) to mitral valve septal annular velocity (*e′*) ratio. *Control* ($n = 5$), *CD* cardiac disease ($n = 3$), *RD* renal disease ($n = 5$), *CRD* cardiorenal disease ($n = 4$)

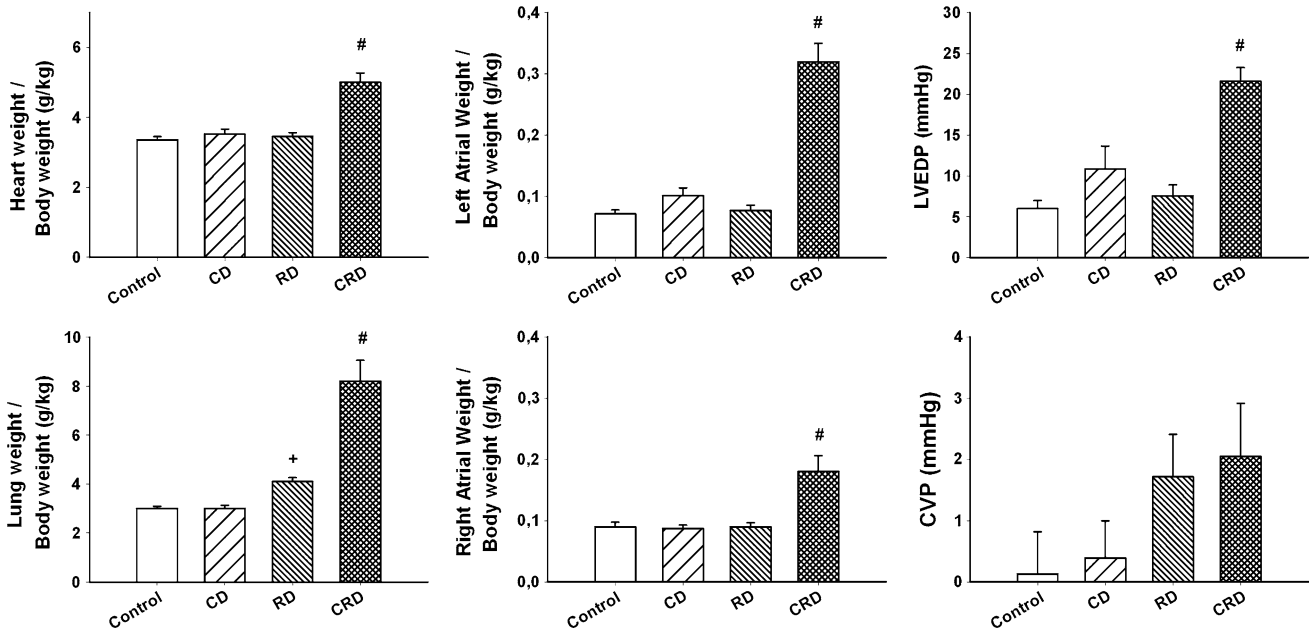

Fig. 3 Parameters of congestive heart failure. *Control* ($n = 10$), *CD* cardiac disease ($n = 6$), *RD* renal disease ($n = 9$), *CRD* cardiorenal disease ($n = 10$). *LVEDP* left ventricular enddiastolic pressure, *CVP* central venous pressure. $^{+}$Significant effects of renal dysfunction; $^{\#}$ Significant effect of combined cardiorenal dysfunction

depressed in both groups with MI, and remained at this level for the rest of the study. CO (Fig. 2b) showed similar and stable values in sham rats of both strains. Three weeks after MI, CO was strongly depressed and remained low in CRD, but was partly restored in CD.

Cardiac function and hemodynamics parameters measured 12 weeks after surgery, are presented in Table 3. Heart rate was similar in control rats, rats with CD and rats with RD, but higher ($p = 0.06$) in rats with CRD. RD rats had higher MAP than control, which was significantly reduced after MI. MI reduced left ventricular systolic pressure by 4% in CD, but by 20% in CRD. Whereas MI-induced reduction of left ventricular systolic function ($+dP/dt$), was not exaggerated in CRD compared to CD, diastolic dysfunction, presented as a reduction of relaxation velocity ($-dP/dt$) as well as increase in tissue Doppler parameter (E/e'), was significantly more pronounced in CRD (Fig. 2c). Moreover, heart weight–body weight ratio, as well as lung weight–body weight ratio was significantly higher in the CRD rats (Fig. 3). Parameters of congestion, upstream from the injured left ventricle were analyzed and presented in Fig. 3. LVEDP was increased by 80% in CD, but almost by 200% in CRD, indicating elevated left ventricular preload. Similarly, left atrial weight increased 3 times more in CRD than in CD. Twice as high right atrial weight only in CRD rats suggests that congestion was not restricted to the damaged left side of the heart. In addition, central venous pressure appeared highest in the CRD rats, although no significant differences could be obtained between the experimental groups.

Effects on myofilament function

Myofilament function of isolated skinned fibers from the viable left ventricular free wall is presented in Fig. 4. Whereas active force was similar in all groups, passive force increased after MI. This increase was found statistically significant in CRD, but not in CD.

Effects on cardiac structure

Histological analysis of left ventricle of 25 weeks old sham rats revealed similar effects to those of 12 weeks old rats (Fig. 5). MI significantly reduced capillary density to the same extent in CD and CRD. Similarly, interstitial collagen and myocyte size were increased due to MI, but without differences between CRD and CD. Furthermore, no differences between the groups are found for number of capillaries per myocyte (range 1.8–2.3).

Myocyte size in the right ventricle was significantly and exponentially ($p < 0.001$) increased in rats with CRD (Fig. 5), and was significantly and positively correlated with congestion parameters, such as LVEDP, atrial weight and lung weight. Hence, right ventricular myocyte size may better explain the increased heart weight body weight ratio ($r^2 = 0.418$; $p < 0.001$) in rats with CRD than left ventricular myocyte size ($r^2 = 0.002$; $p = 0.797$).

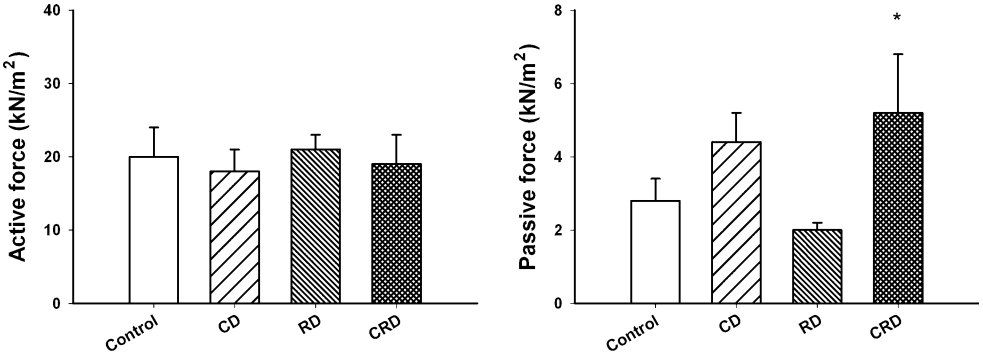

Fig. 4 Active and passive force development in isolated myofibrils. *Control* ($n = 5$), *CD* cardiac disease ($n = 5$), *RD* renal disease ($n = 5$), *CRD* cardiorenal disease ($n = 5$). *Significant effect of cardiac dysfunction

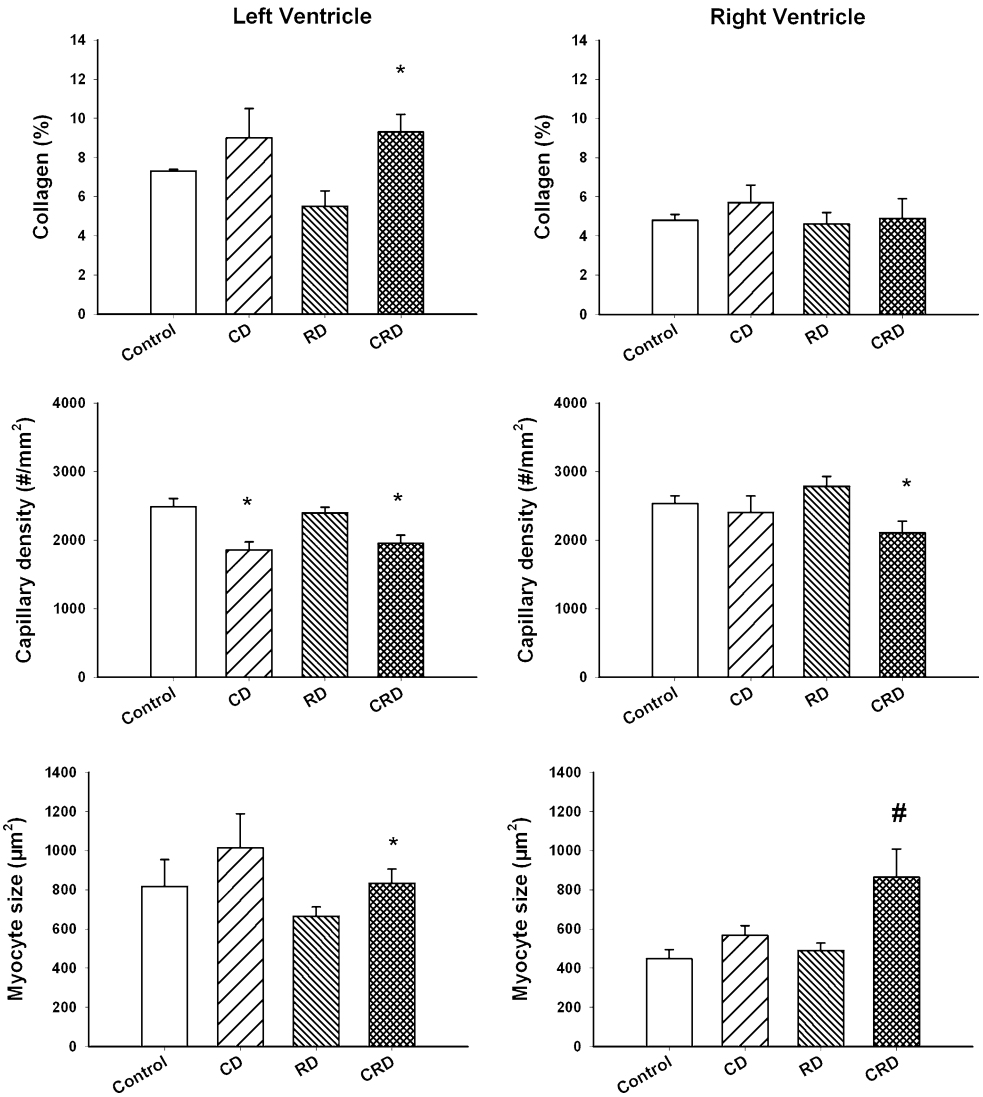

Fig. 5 Left and right ventricular remodeling presented as interstitial collagen, capillary density and myocyte hypertrophy. *Control* ($n = 10$), *CD* cardiac disease ($n = 5$), *RD* renal disease ($n = 9$), *CRD* cardiorenal disease ($n = 10$). *Significant effects of cardiac dysfunction; #Significant effect of combined cardiorenal dysfunction

Circulatory effects

Effect on blood

Before MI, plasma renin activity (PRA) was significantly lower in RD compared to control rats; 4.4 ± 0.5 versus 8.0 ± 0.8 ngAngI/ml/h. MI induced a significant increase in PRA only in CRD rats (altered PRA from before- to 7 weeks post-MI: CRD +6.2 ± 1.6; CD −2.1 ± 2.5; RD −1.2 ± 1.2; control −2.5 ± 1.0 ngAng I/ml/h). This increased PRA was significantly correlated with LVEDP ($r^2 = 0.461$, $p < 0.001$), lung weight ($r^2 = 0.515$,

$p < 0.001$) and right ventricular myocyte size ($r^2 = 0.175$, $p = 0.042$), but not with renal parameters, such as proteinuria, creatinine and renal blood flow.

Before MI, there was no difference in BNP levels between groups. MI induced highest increase of BNP in CRD rats (altered BNP from before to 7 weeks post-MI: CRD 1.1 ± 0.2; RD 0.3 ± 0.4; CD 0.5 ± 0.2; control 0.6 ± 0.2 ng/ml). BNP values 7 weeks post-MI were positively correlated with lung ($r^2 = 0.184$, $p = 0.014$) and atrial weights ($r^2 = 0.215$, $p = 0.008$ and $r^2 = 0.138$, $p = 0.040$ for left- and right atrium, respectively), but not with renal parameters.

MI in CD did not affect hematocrit, nor number of red blood cells (Table 4). However, number of white blood cells was significantly reduced after MI. In RD, lower hematocrit levels shown at 12 weeks of age, were also observed at 25 weeks, and matched with lower numbers of red blood cells (Table 4). Number of white blood cells was pronouncedly reduced in RD. In CRD, MI did not affect hematocrit, number of red blood cells, or number of white blood cells. Platelet numbers were similar in all groups.

Vascular effects

There were no differences between groups with regard to aortic media/lumen ratio at 12 weeks after MI (control 0.23 ± 0.03; CD 0.23 ± 0.04; RD 0.25 ± 0.2: CRD 0.28 ± 0.02). There was a slight increase in total peripheral resistance in CD rats from 0.71 ± 0.07 to 0.95 ± 0.10 mmHg min/ml, and in CRD from 0.81 ± 0.07 to 1.04 ± 0.18 mmHg min/ml. A significant correlation was found between TPR and left ventricular congestion parameters, LVEDP and left atrial weight ($r^2 = 0.328$, $p = 0.026$; $r^2 = 0.355$, $p = 0.016$, resp.), but not with pulmonary and right ventricular congestion parameters, nor with renal parameters.

In aortic rings, endothelium-independent relaxation, obtained by response to sodium nitroprusside, was reduced by 23% (NS) in CD and RD and by 53% in CRD rats ($p < 0.05$). Moreover, endothelium-independent relaxation was significantly correlated with congestion parameters LVEDP, left atrial weight and lung weight, but not with any of the renal parameters.

Endothelium-dependent relaxation, obtained by the response to acetylcholine, is presented in Fig. 6a. RD caused a significant reduction in endothelium-dependent relaxation (AUC control 99.0 ± 14.8 AU; RD: 54.6 ± 11.9 AU), which was maintained after correction for endothelium-independent relaxation. CD did not significantly impair endothelium-dependent relaxation (AUC 93.0 ± 37.8 AU). Although not statistically significant, an additional effect might be seen in the vessels from CRD rats (AUC 32.8 ± 9.4 AU). Interestingly, endothelium-dependent relaxation was significantly correlated with both renal dysfunction parameters as well as left ventricular overload parameters (Fig. 7), but not with systemic hemodynamic parameters, such as TPR, MAP and CVP.

Endothelial NO-synthase expression was slightly increased in aortas of rats with renal dysfunction, but without additional effects of MI (eNOS/Rplp0 in control 0.82 ± 0.15; CD 0.75 ± 0.27; RD 1.11 ± 0.20; CRD 1.18 ± 0.17).

Effects of COX inhibition on Ach-induced relaxation is presented in Fig. 6b. In control as well as RD, COX2 inhibition by nimsulide enhanced the Ach-induced relaxation, whereas COX1 and COX2 inhibition by indomethacin had no effect, indicating a COX1 mediated vasodilation compensated by COX2 mediated vasoconstriction. MI had similar effects in CD as well as CRD; no effect of nimsulide, but increased vasorelaxation on indomethacin.

Discussion

Even mild renal dysfunction substantially increases the risk of cardiovascular morbidity or mortality, and many patients with heart failure suffer from concomitant renal dysfunction, supporting a strong interaction between heart and kidneys. Primary chronic kidney disease is associated with extremely high cardiovascular risk [36].

Table 4 Effects on hematocrit (Hct), number of red- (RBC) and white (WBC) blood cells, and platelets

Group	Control	CD	RD	CRD
N	10	5	9	9
Hct (%)	47.8 ± 0.6	48.7 ± 1.0	45.0 ± 0.8[b]	45.7 ± 0.7[b]
RBC (#.10^{12}/l)	9.4 ± 0.1	9.4 ± 0.1	7.9 ± 0.2[b]	7.8 ± 0.1[b]
WBC (#.10^{12}/l)	10.7 ± 0.8	8.7 ± 0.6[a]	2.2 ± 0.2[b]	2.3 ± 0.1[b]
Platelets (#.10^{12}/l)	739 ± 68	852 ± 49	887 ± 44	832 ± 40

CD cardiac disease, *RD* renal disease, *CRD* cardiorenal disease, # number

[a] Significant effects of cardiac dysfunction

[b] Significant effect of renal dysfunction

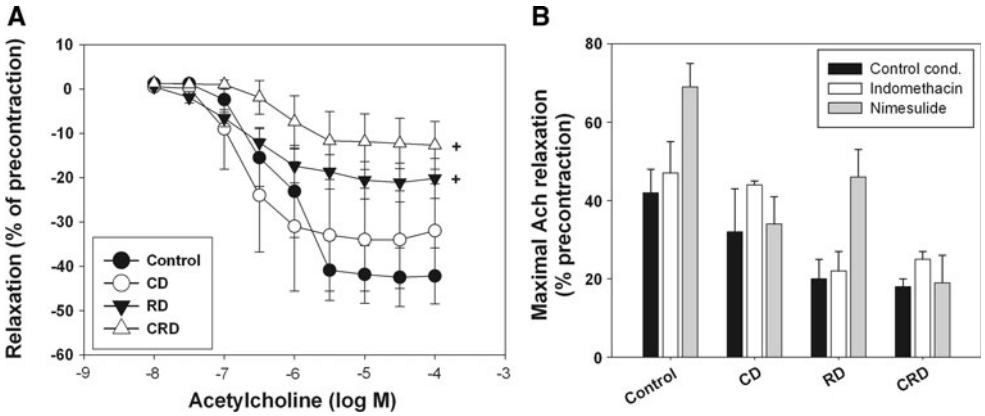

Fig. 6 Parameters of endothelial function. **a** Concentration-dependent relaxation to acetylcholine in phenyleprine-precontracted aortic rings. **b** Effect of COX inhibition on maximal Ach-induced relaxation. *Control* ($n = 6$), *CD* cardiac disease ($n = 4$), *RD* renal disease ($n = 6$), *CRD* cardiorenal disease ($n = 5$). *CTRL* control conditions, *INDO* indomethacin, *NIM* nimesulide; $^{+}$Significant effect of renal dysfunction

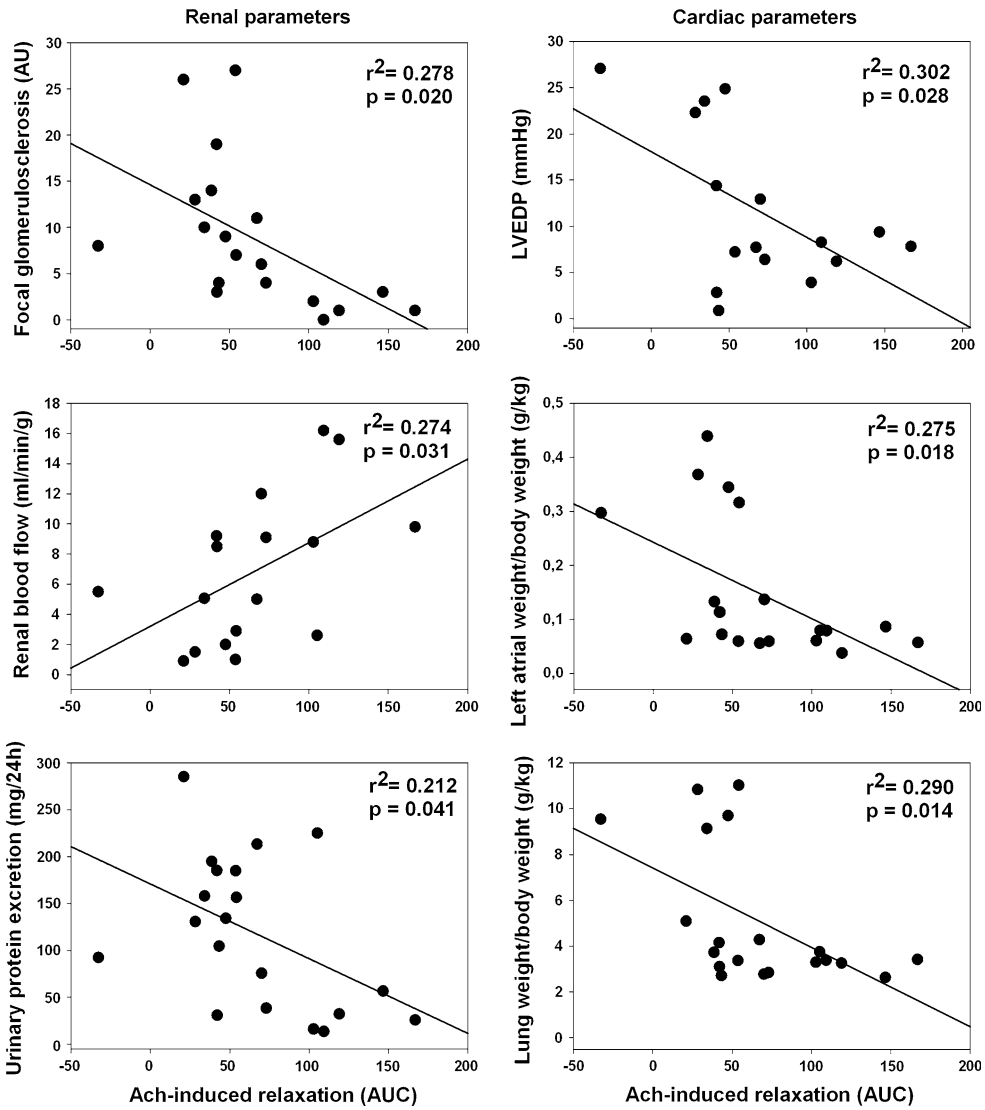

Fig. 7 Statistically significant correlations between endothelial function and renal as well as cardiac parameters

The interaction between heart and kidneys in this relatively high-risk group of patients was studied in an animal model mimicking the clinical condition; rats that spontaneously develop progressive renal dysfunction, subjected to experimentally induced heart failure. The main findings were: (1) cardiac, but not renal dysfunction was exaggerated in combined cardiorenal dysfunction; (2) cardiac dysfunction was characterized by systolic and diastolic

dysfunction with cardiopulmonary congestion and right ventricle hypertrophy; (3) adverse left ventricular remodeling and myofilament dysfunction could not explain the deteriorated cardiac function in cardiorenal disease; (4) vascular endothelial dysfunction may provide a link between renal and cardiac dysfunction.

Rat model for cardiorenal disease

As anticipated from literature [35], renal dysfunction in 12 weeks old RD rats was confirmed by functional, structural and hemodynamic changes, represented by increased UPE and FGS and reduced renal blood flow, respectively. Furthermore, hematocrit levels were reduced, which is in agreement with previous findings in rats [1], as well as in patients with chronic renal disease [23, 46], and notified as a "novel" risk factor in cardiorenal disease [47]. RD coincided with mildly increased MAP and normal CVP [24].

Combining this model with experimental myocardial infarction, a well-established model of cardiac dysfunction [37, 38], may therefore provide a suitable animal model to study chronic renal dysfunction as predisposition for high cardiovascular risk.

Renal dysfunction in cardiorenal disease

There was no effect of cardiac dysfunction on progression of renal dysfunction as evidenced by functional (UPE), hemodynamic (RVR) and structural (FGS) parameters. Apart from the initial decrease, the progression of UPE in CRD was similar to that in RD. Since this initial decrease occurred to the same extent in CD and CRD rats, it might be attributed to reduction in MAP shortly after MI. The absence of progressive proteinuria is in contrast to results reported in rats after unilateral nephrectomy and MI [48], but in accordance with the commonly used cardiorenal model of rats with subtotal nephrectomy and MI [55, 56]. Renal dysfunction in RD and CRD resulted in increased FGS in comparison to control and CD; however, it was lower in CRD than in CD. When taking the strong correlation between UPE and FGS, it seems that this difference may have also occurred in the early post-MI phase. RVR was severely increased in RD compared to control, but without additional increase in CRD. All these functional, hemodynamic and structural parameters of renal dysfunction were significantly inter-correlated, supporting mutual interaction of renal parameters and substantiating the observation of absence of progressive renal dysfunction due to concomitant cardiac dysfunction.

In contrast, creatinine clearance, as an estimate of glomerular filtration rate, was reduced over time, but not different between experimental groups. This would support recent suggestions that proteinuria and reduced glomerular filtration rate may represent different types of renal dysfunction, rather than being a measure of increased severity [5]. Finally, no correlations were found between renal parameters and cardiac parameters, indicating absence of a direct relationship in the present study.

Although not accelerated by concomitant cardiac dysfunction, renal function still was declining over time in CRD. It is generally acknowledged that worsening of renal function can lead to worsening of cardiac function and outcome in patients, as reviewed by Damman and coworkers [6]. However, to our knowledge there are no clinical studies available investigating the opposite relation: comparing worsening of renal function in patients with or without concomitant cardiac dysfunction.

Cardiac dysfunction in cardiorenal disease

Calculated CO provided a first indication of more severe cardiac dysfunction in rats with primary renal dysfunction. Accelerated cardiac dysfunction in CRD, as indicated by lower CO, was substantiated by exponential increases in left ventricular overload parameters, congestion and failure. Comparing our results to available literature reveals that accelerated cardiac dysfunction was not observed in unilateral nephrectomy + MI [48] or in 5/6 nephrectomy + MI [56], whereas rats with temporal L-NNA infusion, in addition to 5/6 nephrectomy, did show signs of more severe cardiac dysfunction [1]. However, one recent study is showing aggravation of cardiac remodeling with concomitant renal dysfunction [10]. Unfortunately, this study did not include rats with only 5/6 nephrectomy or sham MI rats. Moreover, control rats did not show cardiac dysfunction after MI as measured by EF or LVEDD, and changes in cardiac function and remodeling could well be attributed to the larger infarct sizes in the cardiorenal group [10].

Since the congestion in systolic heart failure is related to renal dysfunction and increased mortality [7], many studies focused on systolic dysfunction. However, in a substantial proportion of cardiorenal disease patients, cardiac dysfunction is characterized by diastolic rather than systolic dysfunction; heart failure with preserved ejection fraction [29, 32]. Data from the present study indicate that rats with CRD may display diastolic heart failure indicated by stronger reduction in cardiac output at similar ejection fraction, increased *E/e*' ratio and signs of congestion. This was accompanied by right ventricular overload, indicative for pulmonary hypertension, as commonly observed in cardiorenal diseased patients [26]. In a recent study, it was argued that diastolic dysfunction only develops into congestive diastolic heart failure because of underlying renal

insufficiency [51], which underscores the relevance of our model.

Underlying mechanisms

Adverse cardiac remodeling

In 12 weeks old MWF, a cardiac phenotype was indicated from higher heart weight–body weight ratios associated with increased left ventricular systolic pressure at normal end-diastolic pressure. Three months of CRD resulted in pronouncedly increased cardiac weight, suggesting adverse cardiac remodeling [43]. However, besides a minor, and possibly blood pressure-related left ventricular myocyte hypertrophy, adverse remodeling of the left ventricle was not observed either at 12 or at 25 weeks. Thus, adverse left ventricular remodeling neither was a predisposition to develop accelerated cardiac dysfunction in CRD, nor could be associated with the exaggerated cardiac dysfunction in CRD, once established. This is in accordance with results in rats with 5/6 nephrectomy + MI [34, 56], but in contrast to a recent study of Dikow et al. [10]. In this latter study, however, effects on remodeling were only observed in the border zone of the scar and not in the remote areas, and hence would match with our results obtained in remote areas.

In the present study, increased pulmonary weight suggested pulmonary congestion and right ventricular overload. This was evidenced by a significant increase in right ventricular myocyte size, indicating right ventricular concentric hypertrophy, and could be associated with the activated renin angiotensin system in CRD. A higher correlation between total ventricular weight and right-, compared to left ventricular myocyte size, supports an important contribution of the right ventricle to increased heart weight in CRD. Moreover, right ventricle overload might have also contributed to the dysfunction of LV [4].

Myofilament function

Cardiac diastolic dysfunction could be caused by various mechanisms [11], including altered function of contractile proteins within the cardiomyocyte. Whereas active force was similar in all groups, passive force was significantly increased in CRD. Correlation with impaired left ventricular relaxation indicated intrinsic diastolic dysfunction rather than fibrosis-related stiffness of the left ventricle. High cardiomyocyte stiffness has been reported in diastolic dysfunction patients and correlated significantly with LVEDP [2].

Anemia

Irrespective of concomitant cardiac dysfunction, MWF showed anemia, indicated by lower hematocrit as well as number of red blood cells. Since white blood cell number is even more pronouncedly decreased, anemia may result from general bone marrow dysfunction rather than from hemodilution or erythrocyte-specific mechanisms, such as erythropoietin-resistance [23]. This is supported by results of a pilot study of culturing bone marrow-derived endothelium progenitor cells [33], showing less than 10% yield in MWF compared to Wistar. All aspects, including anemia [23, 25], erythropoietin-resistance [39], reduced number/and or function of endothelium progenitor cells [3, 16, 40], general bone marrow dysfunction [21, 22], are commonly described in renal dysfunction. However, as no additional effects were observed in CRD rats compared to CD and RD rats, this does not provide an explanation for the accelerated cardiac dysfunction in primary renal dysfunction.

Vascular function

Endothelial dependent relaxation measured in aortic rings served as a biomarker for endothelial dysfunction in more distant vascular beds. MI has been associated with reduced endothelium-dependent relaxation in aortic rings [13, 53]. Moreover, impaired endothelium-dependent relaxation in aortic rings in MWF [44] could be associated with endothelial dysfunction in coronary, but not in mesenteric arteries [15]. There is growing evidence of the role of endothelium in regulation of cardiac diastolic function [54]. It is believed that low availability of NO produced by endothelial NO synthase leads to decrease in diastolic function, cardiac hypertrophy and fibrosis, whereas enhancement of this enzyme attenuates these changes. Endothelium derived NO has been shown to have positive effects not only on diastolic, but also on systolic function [18]. Moreover, the endothelial dysfunction might limit the cardioprotective effect of erythropoietin [42]. Therefore, exaggerated endothelial dysfunction in CRD might have contributed to further impairment of cardiac dysfunction in these rats. However, as eNOS expression in the present study was similar in RD and CRD and even slightly higher compared to control and CD, impaired eNOS would not explain the endothelial dysfunction in CRD. Similarly, MI-induced altered COX-activity was comparable in CD and CRD and hence may not have contributed to endothelial dysfunction in CRD. Unfortunately, to our knowledge no data are available about endothelial function in renal arteries in MWF. Interestingly, aortic endothelial function was the only parameter that significantly correlated with renal as well as cardiac parameters, suggesting a mediating role for the vasculature. This suggested role may be supported by the finding that endothelial function predicts individual susceptibility to renal damage in 5/6 nephrectomy [14, 30, 31].

Study limitations

Although the present study may throw new light on the interaction between heart and kidneys in cardiorenal disease, it may have some limitations as well: the MWF rat strain used in this study is an inbred model based on proteinuria, which could have caused other unknown genotypical/phenotypical changes as well; we cannot fully exclude primary pulmonary disease in this strain. Moreover, as most inbred Wistar-origin models, MWF rats had substantially lower body weights at the same age than outbred Wistar. Although a clear explanation is to our knowledge not known, it may trouble either/not correcting for body weight. Furthermore, although proteinuria, focal glomerulosclerosis, mild hypertension and reduced renal blood flow are well known in the MWF's, reduced GFR as often seen in cardiorenal patients was not present in the MWF. Finally, the first indication for exaggerated cardiac dysfunction came from echocardiographical measurements only performed in a subgroup of rats, which clearly hampers proper statistical analysis.

Conclusion

In conclusion, experimental MI did not exaggerate renal dysfunction in the proteinuric MWF rat. However, cardiac dysfunction was exaggerated by this primary renal dysfunction. The accelerated cardiac dysfunction could be characterized by elevated left ventricular preload, as well as pulmonary congestion, right ventricular overload and right ventricular hypertrophy. This animal model may provide an important step to understand the complex clinical condition of cardiorenal disease. Finally the study provides evidence that vascular endothelial dysfunction may play a mediating role in this cardiorenal interaction.

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Conflict of interest None.

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